Measurement of IgA activity against parasitic larvae, fecal egg count and growth rate in naturally infected sheep

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Abstract

Gastrointestinal parasitism is one of the greatest causes of disease and lost productivity in domestic animals. Anthelmintic treatment is the mainstay of current control methods. However, with the widespread use of anthelmintics, the problem of parasite resistance has emerged. Other modalities of prevention and treatment are urgently needed. One of the most promising developments in this respect is the use of genetically resistant sheep. Selective breeding of animals resistant to gastrointestinal parasitism is particularly attractive, but identifying the phenotypic and genetic markers of resistance on which selection will be based is a major problem. Hence, a study was undertaken to investigate some of the phenotypic determinants of resistance to nematode infection in Scottish Blackface sheep when naturally infected with the gastrointestinal nematodes, particularly Teladorsagia circumcincta, and how these determinants may facilitate the successful selection of resistant animals. The present study has not found any correlations between plasma IgA activity against third-stage larvae of T. circumcincta and fecal egg. Moreover, there was no significant correlation between growth rates in 24 week old lambs. The results suggest that IgA activity against fourth-stage larvae is probably a better marker of nematode resistance than IgA activity against third-stage larvae. The work detailed here has further increased our understanding of the complex host/parasite relationship, and has confirmed that selective breeding, using the various phenotypic markers.

Key words: IgA activity, Teladorsagia circumcincta, Host-Parasite relationship

Introduction

Gastrointestinal nematode parasitism is arguably the most serious constraint affecting sheep production worldwide (6). Of the novel approaches to parasite control, breeding sheep for resistance seems to be the most promising application that can be used to complement the strategic use of dewormers and improved pasture management (2). Among gastrointestinal nematode infection, Teladorsagia circumcincta is important in Scottish Blackface sheep. T. circumcincta has a direct life cycle (no intermediate host) and involves development through a series of cuticle shedding molts. There are three post-hatching pre-parasitic stages L1, L2 and L3, and two parasitic stages L4 and a final L5 immature adult stage. Hatched larvae become infective (L3 larvae) in about 6 to 7 days and most larvae survive on pasture for 4 months, although some larvae may survive much longer. Infection is by ingestion of L3 larvae by the host while grazing. The L3 larvae penetrate the lumen of the abomasal gland and moult to the L4 stage. Following
a period of growth and a further moult to the L₅ (immature adult) stage, they emerge from the gland and mature on the mucosal surface. Male and female worms copulate producing eggs, which are passed by the host in the faeces. The eggs hatch to the L₁ stage and further development to the L₂ and L₃ stages on pasture. Typically this life cycle takes three weeks to complete with variation depending on the weather and immune status of the animal (14). Anthelmintics have a strong effect in limiting worm burdens, and in particular in controlling adult worms. However, eggs can be found in faeces within 14-28 days after drug treatment coming from fresh infections or from worms that survive treatment. The development of resistance to anthelmintics is a major threat to parasite control worldwide (15).

Nematodes cause disease but perhaps their major economic impact is the reduction in growth of young lambs. Due to the emergence of anthelmintic resistance in parasite population, non-chemotherapeutic methods are being investigated to control T. circumcincta infection (1). A detailed understanding of the immune emergence of anthelmintic resistance in parasite populations, mechanism involved in resistance to infection will lead to more sustainable methods of control and in particular will aid in the identification of resistant animals.

Fecal egg count has been widely used as an indicator of host resistance to gastrointestinal parasites in sheep and has been shown to be a heritable trait (7). The strength of the association between IgA and parasite fecundity led to hypothesis that the specificity and activity of local IgA was the major mechanism regulating the fecundity of T. circumcincta (11) and a major mechanism of resistance to infection in lambs. A comparison of IgA response to third stage, fourth stage and adult T. circumcincta indicated the strongest association with reduced worm length, with increased response to fourth stage larvae (12). The response of the third stage larvae were correlated with the response to fourth stage larvae (10). IgA activity against fourth stage larvae probably is the best phenotypic marker, but recovering fourth stage larvae requires killing sheep. Third stage larvae can be recovered from fecal cultures, making responses to third stage larvae cheaper and easier to measure and killing animals can be avoided. There appear to be very few studies that have examined IgA activity against third stage larvae as a phenotypic marker for resistance in naturally infected sheep.

The present investigation was undertaken to determine the relationships between IgA activities against third stage larvae of T.circumcincta, fecal egg counts and growth rate of Scottish Blackface lambs and to explore whether plasma IgA activity against third stage larvae could be used as indicator trait for resistance or susceptibility in sheep.

Materials and Methods

Animals: Seven hundred and fifty nine naturally infected six month old Scottish Blackface lambs were sampled for blood and feces in October for a period of three years.

Fecal Worm Egg Count: Feces samples were taken directly from the rectum of the lambs and stored at 4°C until processed. A modified McMaster salt flotation technique (5, 16) was performed to estimate the concentration of nematode eggs in the feces.

Weight of the lambs: Individual body weight of the experimental lambs was recorded using a sheep weighing scale at each blood sampling and fecal collection dates.

Preparation of third stage larvae: Third stage larvae were collected from fecal cultures of eggs of deliberately infected sheep and exsheathed in 1% Sodium hydrochloride in phos-
phosphate buffered saline (PBS, pH 7.4) for 10 minutes at 37°C. Exsheathed larvae were then resuspended in 50 ml PBS and centrifuged at 100g for 10 minutes. The supernatant was removed and then washed twice. The larval pellet was washed once in PBS containing 100 IU ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 2.5 μg ml⁻¹ amphotericin B and 0.05 mg ml⁻¹ gentamicin to prevent any contamination (10). Larvae were then given a final wash in 50 ml of 10 mM Tris buffer (pH 8.3), containing 1mM disodium ethylene diamine tetracetic acid (EDTA), 1mM ethylene glycol bis (2-amminesulfo ethyl ether)-N,N,N',N'-tetracetic acid (EGTA), 1mM N-ethylmaleimide (NEM), 0.1 μg pepstatin, 1mM phenyl methyl sulphonyl fluoride (PMSF), and 0.1 mM N-tosylamide-L-phenylalanine chlormethyl ketone (TPCK) as protease-inhibitor solution containing 1 % sodium deoxycholate and homogenized using a handled electric homogenizer on ice. When larvae were completely homogenized, they were centrifuged at 500g for 20 minutes and the soluble extract was filtered through a sterile 0.2µm syringe filter. The extract was again spun at 500g for 20 minutes and the supernatant aliquoted and stored at -80°C.

**Blood Samples:** Blood samples were collected by jugular venepuncture into evacuated glass tubes containing 20mM disodium EDTA as an anticoagulant. Plasma and buffy coats were obtained by centrifugation at 100g for 30 minutes and stored at -20°C for further use.

**Enzyme-Linked Immunosorbent Assay (ELISA):** ELISA was used to detect Parasite specific host plasma IgA activities to infection against somatic larval extracts of third stage larvae from *T. circumcincta*. Each well of a 96-well flat-bottomed microtitre plate (Nunc) was coated with 100 μl of larval antigen preparation (L₃) at 5μg ml⁻¹ in 0.06M sodium carbonate buffer pH 9.6 overnight at 4°C. The plates were then washed five times in PBS-T. The individual sheep plasma samples were diluted 1:10 in PBS + 0.4% skimmed milk (PBS-TSM). The plates were then incubated with 100 μl per well, in duplicate for 30 minutes at 37°C. Positive and negative controls were diluted 1:10 in PBS-TSM and run in triplicate on each plate to minimize the effect of variation between plates on different days. Plates were incubated with 100 μl per well of a monoclonal rat IgG anti-sheep IgA diluted in PBS-TSM for 30 minutes at 37°C. After five washes in PBS-T, the plates were incubated with 100 μl per well of a mouse anti-rat IgG alkaline phosphatase antibody conjugate (Sigma) diluted in PBS-TSM for 30 minutes at 37°C. After a final five washes in PBS-T, the plates were incubated in 100 μl per well with Bluephos® Microwell Phosphatase Substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 37°C. The optical density of each well was then read at 635 nm with a multichannel spectrophotometer (Titertek Multiscan MC, Labsystems, Oy, Finland or Dynex MRX, Dynex Technologies, Ashford, UK) for 15 minutes. Usually this was done at five minute intervals, until the positive control optical density reading was between 1.5-2. Each batch of monoclonal and secondary antibody was titrated to determine the appropriate concentration. This procedure was carried out on the Grifols Triturus® ETA Analyser.

**Optical density indices:** The Positive and negative controls in each ELISA assay were pooled plasma samples from individual animals which had given either very strong (positive control) or very weak (negative control) optical densities using the methods of Sinski et al. (10). To minimize the variation between results obtained on different days and between plates, optical densities for each sample were transformed into an optical density (OD) index (10).

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Statistical Analysis: The association between IgA activity, fecal egg counts and growth rate was estimated by correlation coefficient using the correlation procedure in the SAS statistical package (SAS Institute, Cary, NC, USA). Egg counts were transformed prior to statistical analysis by taking the logarithm of the egg count plus 10.

Results

IgA activity against an extract of third-stage larvae was measured using an indirect ELISA and expressed as a percentage of a standard value. Fig. 1 shows the regression between replicate measurements of IgA activity in October during the first year. The two measurements were carried out to evaluate the repeatability between ELISAs with the same samples on different dates. The regression shows that the two measurements gave similar results. The correlation between the first and the second run was positive and very highly significant ($r = 0.80, p < 0.001$).

![Fig. 1 Regression between replicate measurements of IgA activity against third-stage larvae.](image)

The distribution of IgA activities against third-stage larvae was positively skewed in each year; most lambs had relatively low values but some lambs had quite high values. Fig. 2 shows the distribution of IgA activity against third-stage larvae of T. circumcincta as measured by simple indirect ELISA in the lambs sampled in October during the three years of study.

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The number of animals, mean IgA activity, standard error of mean, minimum and maximum value observed in October during the period of study are given in Table I. The mean plasma IgA activity was 0.11 in first year, 0.09 in second year and 0.19 in third year. Mean IgA activity varied among years but the standard errors are quite small. The mean IgA optical density indices ranged from 0-1.02 in the first year, 0-1.24 in the second year and 0-1.12 in the third year.

**Table 1.** Mean and standard error of IgA activity against third-stage larvae of *T. circumcincta*

<table>
<thead>
<tr>
<th>Date of sampling</th>
<th>Number of animals</th>
<th>Mean of IgA activity</th>
<th>Std. Error of mean</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 year-October</td>
<td>236</td>
<td>0.108</td>
<td>0.008</td>
<td>0.000</td>
<td>1.02</td>
</tr>
<tr>
<td>2 year-October</td>
<td>261</td>
<td>0.091</td>
<td>0.007</td>
<td>0.000</td>
<td>1.24</td>
</tr>
<tr>
<td>3 year-October</td>
<td>262</td>
<td>0.186</td>
<td>0.000</td>
<td>0.000</td>
<td>1.12</td>
</tr>
</tbody>
</table>

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Table 2. Eggs per gram of feces in lambs sampled during three years

<table>
<thead>
<tr>
<th>Date of sampling</th>
<th>Mean</th>
<th>Std. Error of Mean</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 year October</td>
<td>278.2</td>
<td>19.1</td>
<td>0</td>
<td>1700</td>
</tr>
<tr>
<td>2 year October</td>
<td>181.6</td>
<td>13.4</td>
<td>0</td>
<td>1200</td>
</tr>
<tr>
<td>3 year October</td>
<td>252.7</td>
<td>14.5</td>
<td>0</td>
<td>1238</td>
</tr>
</tbody>
</table>

The mean faecal egg count was 278.2, 181.6 and 252.7 respectively during the three years studied (Table 2).

The Pearson correlation coefficients were calculated from log-transformed fecal egg counts within each year in the 1st year (r = 0.014, p = 0.826), 2nd year (r = - 0.091, p = 0.147) and 3rd year (r = 0.067, p = 0.286) against the log. transformed optical density indices of IgA activity (Table 3). The results confirm that there was no significant correlation between fecal egg counts and IgA activity against third-stage larvae.

Table 3. Correlations between transformed fecal egg counts and IgA activity

<table>
<thead>
<tr>
<th>IgA activity</th>
<th>Log epg 1st Year</th>
<th>Log epg 2nd year</th>
<th>Log epg 3rd Year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.014</td>
<td>-0.091</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Further, there was no significant correlation between IgA activity and growth rate at 24 weeks old lambs for the 1st year (r = - 0.079, p = 0.248), 2nd year (r = - 0.143, p = 0.021) and 3rd year (r = - 0.042, p = 0.503) (Table 4).

Table 4. Correlations between growth rate at 24 weeks old lambs and IgA activity

<table>
<thead>
<tr>
<th>IgA activity</th>
<th>weight in 1st Year</th>
<th>weight 2nd Year</th>
<th>weight in 3rd Year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.079</td>
<td>-0.143</td>
<td>-0.042</td>
</tr>
</tbody>
</table>

Discussion

Plasma IgA activity was measured against third-stage larvae of *T. circumcincta* in naturally infected sheep, using a simple, indirect ELISA. The results show that the distribution of plasma IgA activity against third-stage larvae in all lambs was positively skewed with the majority of lambs having relatively low activity but a minority lambs had quite high activity (Fig 2). The distribution is similar to the distribution of IgA activity against fourth-stage larvae of *T. circumcincta* (13). Serum IgA is dimeric in ruminants and in experimental studies almost totally derived from the gastrointestinal tract (9). The function of IgA has
been thought to be restricted to binding antigens outside the epithelium basal membrane (14). These results suggest that plasma IgA activity against third-stage larvae may provide a window on local IgA responses in sheep.

No attempt has been made to quantify the amount of IgA in plasma samples. The optical density depends upon the amount of IgA present and the avidity and affinity of IgA for the component of the antigen preparation. As the antigen preparations were complex mixtures, any attempt to estimate absolute antibody concentrations would have been tedious and prone to error (10). The mean optical density indices of plasma IgA activity did not show large differences among the values in the three years.

IgA activity in the serum was dependent upon IgA activity in the abomasum and also the number of adult nematodes present in the abomasums (10). During the early phase of the infection parasites suppressed the acute phase response and the complement system of the host (8). In addition, there was a strong positive relationship between responses to third-stage and to fourth-stage larvae. The correlation coefficients ranged from 0.60 to 0.79 (P < 0.001) for parasite-specific IgA, which showed that sheep with strong responses to third-stage larvae tended to have stronger responses to fourth-stage larvae. Correlated results for the same animals could be due to the existence of some shared, or similar, antigens in the different larval stages. Correlations between responses do not necessarily imply similar amounts of antibody. Positive correlations merely imply that animals that gave higher-than-average responses in the first test gave higher-than-average responses in the second test. So far little work has been done to measure the plasma IgA activity against third-stage larvae as a possible marker of resistance to nematode infection in sheep could be used rather than fourth-stage larvae but more research is needed. The experimental results did not reveal any correlations between fecal egg counts or IgA activity against third-stage larvae. In contrast animals with increased IgA activity against fourth-stage larvae had lower faecal egg counts (13). Therefore IgA activity against fourth-stage larvae is probably a better marker of nematode resistance than IgA activity against third-stage larvae.

Other results reported weak correlations between IgA responses to third (r = -0.155) and fourth-stage (r = -0.176) larval extracts and egg counts (3). The reasons for the differences are unknown but may be due to differences in exposure to nematode infection or nutrition. Unfortunately the results came from lambs on commercial farms and could not be investigated further. However, further trials considering different breeds and age groups of lambs at different periods of years at different environmental conditions should be considered. There were no significant correlations between weight gain at 24 weeks old lambs and IgA activity during the years studied. There appear to be no previous published studies that have reported the relationship between IgA activity and growth rate. More research is necessary to examine the relationship between nematode resistance and IgA activity against third-stage larvae. Further experiment would require necropsy of large numbers of naturally infected animals.

References

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